Development and validation of real time quantitative PCR assays for the detection of olive viruses

MAHER AL RWAHNIH
DEBORAH GOLINO

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Overview

- California is the major producer of olive products in the United States with $130 M in sales in 2019 (over 95% of the olives grown in the US)
- Super high density (SHD) hedge plantings maximize economic gains raise concern about increased spread of disease
- Developing certification programs and regulations is important to protect the olive industry
  - Spread of *Xylella fastidiosa* in olives causing olive quick decline syndrome in Italy
  - Failure of new olive farms in California due to Verticillium wilt and olive knot disease

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Olives at FPS

- 37 varieties
- 8 proprietary (5 from Spain imported under 588 CIP)

Public Varieties

- 14 domestic
- 15 imported from Rome, Italy certification program

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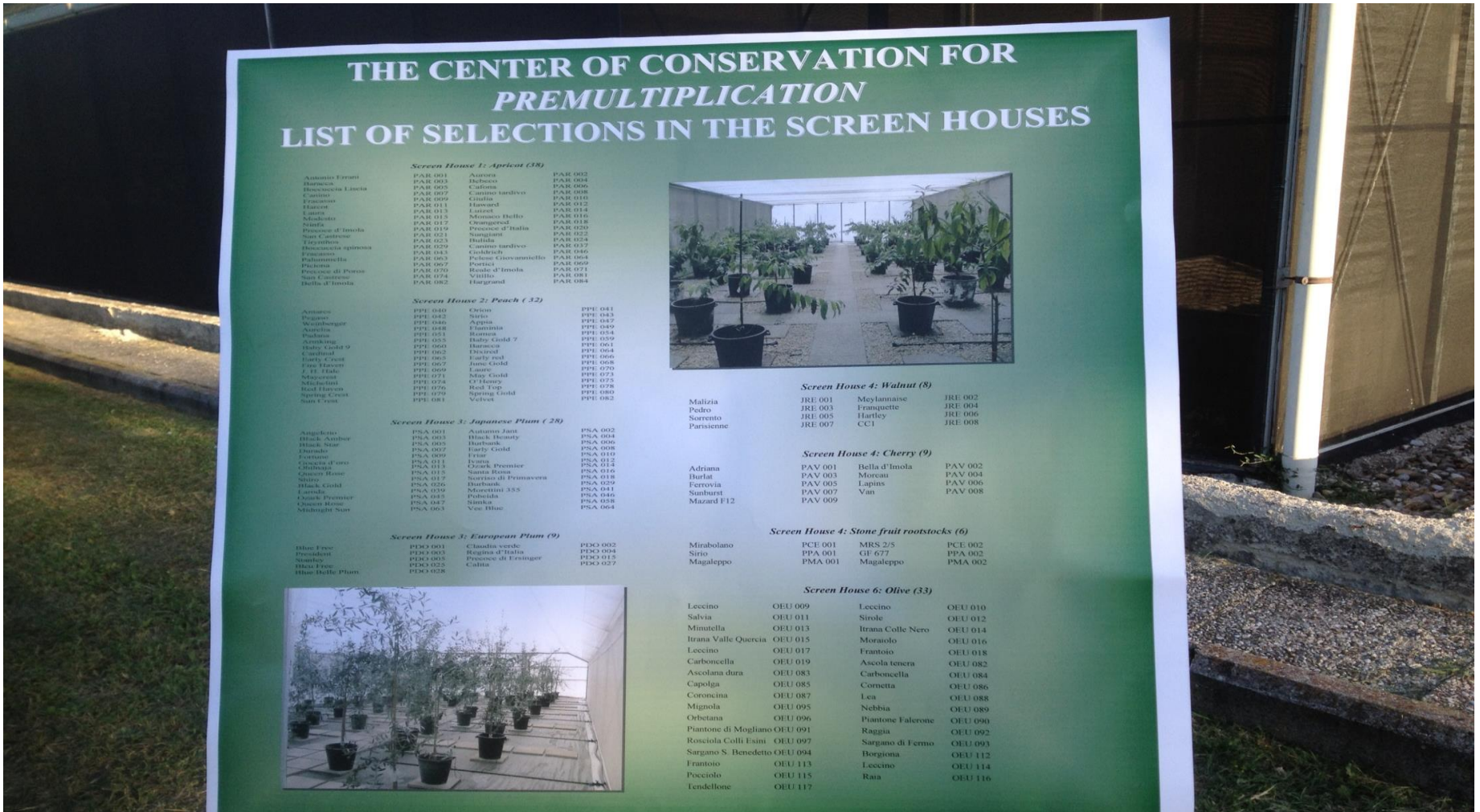
Public Varieties

Arbequina
Arbosana
Ascolana Dura
Ascolano
Borgiona
Cerasuola
Chemlali
Coratina
Cornetta
Coroncina
Frantoio
Hojiblanca
Itrana
Koroneiki
Leccino
Manzanilla
Minutella
Mission
Moraiolo
Nebbia
Nocellara
Picual
Picudo
Pocciolo
Raggia
Salvia
Sargano di Fermo
Sirole
Swan Hill

Olive Certification facilities at the experimental farm of Tor Mancina, Rome-Italy



The National Conservation and Pre-multiplication Center

Olive in Puglia region

(Southern Italy)

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The olive quick decline syndrome in south-east Italy: a threatening phytosanitary emergency

Eur J Plant Pathol (2016) 144:235–243

G. P. Martelli · D. Boscia · F. Porcelli · M. Saponari

Fig. 2 The olive quick decline syndrome: initial (**a**), intermediate (**b**) and final (**c**) stages. Trees in **c** have been heavily pruned, but the new vegetation is already desiccated

- Causal agent: *Xylella fastidiosa. pauca*
- Main vector: *Philanaenus spumarius*
- 1 million infected trees
- Disease eradication and sanitation unfeasible
- Strategies enacted to restrain spread within the currently infected zone

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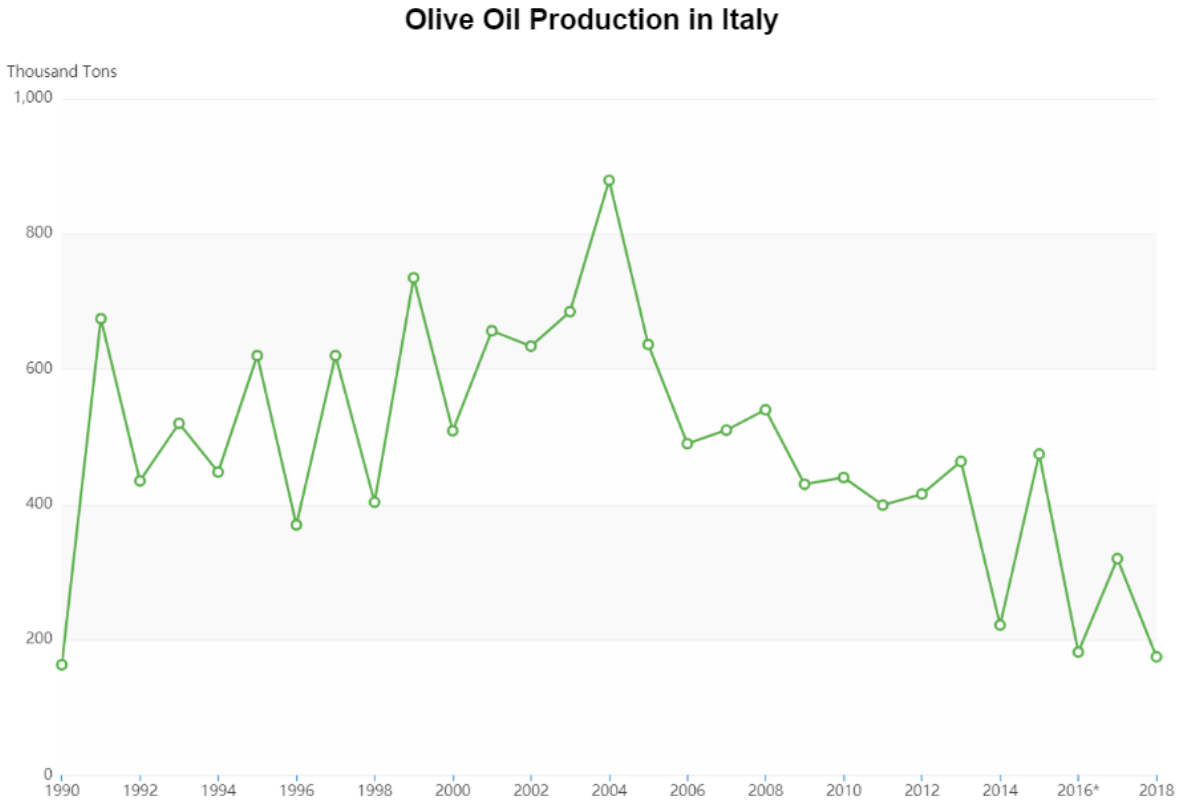
The disease has spread from 19,700 acres, estimated in 2013 to 4,970 square miles in 2020 (DeAndreis 2020, Olive Oil Times)


Ancient Olive Grove

- 59% decrease in 2019



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pathogens

MDPI

Article

The *Xylella fastidiosa*-Resistant Olive Cultivar "Leccino" Has Stable Endophytic Microbiota during the Olive Quick Decline Syndrome (OQDS)

Marzia Vergine [1], Joana B. Meyer [2], Massimiliano Cardinale [1,*], Erika Sabella [1], Martin Hartmann [3], Paolo Cherubini [4,5], Luigi De Bellis [1] and Andrea Luvisi [1]

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The Problem

- Olive is affected by a number of potential or actual pathogens – fungi, bacteria, viruses, unidentified agents (virus-like), and phytoplasmas – that persist in the budwood and can be transmitted and disseminated with it.

- Some of these are agents of recognized diseases, other cause latent infections, whose effect on olive is yet to be determined.

Verticillium wilt

olive knot

root knot nematodes

viruses

phyto-plasmas

X. fastidiosa pauca

Viruses infecting olive in nature

	Virus species	Genus	Geographical distribution (first record)
1	Strawberry latent ring spot virus (SLRSV)	Nepovirus	Italy (1979), Portugal, Spain, USA, Egypt
2	Arabis mosaic virus (ArMV)	Nepovirus	Italy (1979), Portugal, USA, Egypt
3	Cherry leaf roll virus (CLRV)	Nepovirus	Italy (1981), Portugal, Spain, USA, Egypt
4	Cucumber mosaic virus (CMV)	Cucumovirus	Italy (1983), Portugal, Spain, USA
5	Olive leaf yellowing-associated virus (OLYaV)	Closterovirus	Italy (1998), Israel, Lebanon, Egypt, USA
6	Olive latent virus 1 (OLV-1)	Necrovirus	Italy (1984), Jordan, Turkey, Egypt, USA
7	Olive latent ring spot virus (OLRSV)	Nepovirus	Italy (1983), Portugal
8	Olive latent virus 2 (OLV-2)	Oleavirus	Italy (1984)
9	Olive vein yellowing associated virus (OVYaV)	Potexvirus	Italy (1995)
10	Olive yellow mottling and decline associated virus (OYMDaV)	Undetermined	Italy (1995)
11	Tobacco mosaic virus (TMV)	Tobamovirus	Italy (1996)
12	Olive semilatent virus (OSLV)	Undetermined	Italy (1996)
13	Tobacco necrosis virus (TNV)	Necrovirus	Portugal (2002)
14	Olive mild mosaic virus (OMMV)	Necrovirus	Portugal (2005)
15	Olive latent virus 3 (OLV-3)	Marafivirus	Italy (2009)
16	Olea Europaea Geminivirus (OEGV)	Geminivirus	Italy, USA, Spain (2021)

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Bumpy fruits: SLRSV

Cultivar: Ascolana tenera

Reproduction of field symptoms in graft-inoculated rooted cuttings of cv. Ascolana tenera and recovery of SLRSV from symptomatic cuttings were taken as evidence that SLRSV is the causal agent of bumpy fruit (Marte et al., 1998)

Eur. J. Lipid Sci. Technol. 2012, *114*, 535–541

Research Article

Cherry leafroll virus: Impact on olive fruit and virgin olive oil quality

Sara Godena[1], Alessandra Bendini[2], Elisa Giambanelli[3], Lorenzo Cerretani[4], Damir Đermić[5] and Edyta Đermić[6]

- Two olive varieties in Croatian Istria: Frantoio and Ascolana tenera

Findings:

- **Olive quality can be affected in trees with CLRV** - Oils from infected fruits had significant lower value of K232 and K270 and very elevated total phenols content compared to those obtained from healthy olives

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Vein yellowing: OVYaV

Leaf yellowing: OLYaV

OLYaV

OLYaV

- Causes Leaf yellowing
- Can be completly asymptomatic
- 95 % of tested trees are positive for OLYaV

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Journal of Plant Pathology (2011), 93 (1), 227-231 Edizioni ETS Pisa, 2011 227

SHORT COMMUNICATION

CHARACTERIZATION OF LATENT VIRAL INFECTION OF OLIVE TREES IN THE NATIONAL CLONAL GERMPLASM REPOSITORY IN CALIFORNIA

M. Al Rwahnih[1], Y. Guo[2], S. Daubert[1], D. Golino[1] and A. Rowhani[1]

- 49 trees from 36 different cultivars sampled
- All trees asymptomatic
- 98% showed dsRNA profiles indicating viral infection
- 94% positive for olive leaf yellowing-associated virus (OLYaV)
- 35% positive for cucumber mosaic virus (CMV)

Number	Variety	Origin	dsRNA	OLYaV	CMV	Number	Variety	Origin	dsRNA	OLYaV	CMV
1	Arbequina	Spain	+	+	-	26	Midx-elbasan	Unknown	+	+	+
2	Ascolana tenera 1	Italy	+	+	+	27	Mission	Algeria	+	+	+
3	Ascolana tenera 2	Italy	-	-	-	28	Mission 2	Algeria	+	+	-
4	Ascolana dura	Cyprus	+	+	+	29	Mission 3	Italy	+	+	-
5	Azapa	Chile	+	+	+	30	Mission 4	France	+	+	-
6	Barnea	Israel	+	+	+	31	Mission 5	Unknown	+	+	-
7	Bidh El Hammam	Tunisia	+	+	-	32	Mission 6	Italy	+	+	-
8	Bouquetier	Australia	+	+	+	33	Mission 7	Algeria	+	+	-
9	Chalkidiki	Greece	+	+	-	34	Mission 8	Spain	+	+	-
10	Columello	Unknown	+	+	-	35	Mission 9	Unknown	+	+	-
11	Conservolia	Greece	+	+	-	36	Mission Leiva	Unknown	+	+	-
12	Cypress 31	Unknown	+	-	+	37	Mostazal	Unknown	+	+	-
13	Franklin	Unknown	+	+	-	38	Nevadillo	Spain	+	+	-
14	Frantoio	Italy	+	+	-	39	Oblonga	France	+	+	-
15	Gaidourelia	Greece	+	+	-	40	Ogliarola	Italy	+	+	-
16	Grossa di Spagna 1	Italy	+	+	+	41	Rouget	France	+	+	+
17	Grossa di Spagna 2	Italy	+	-	-	42	San Francesco	Italy	+	+	-
18	Grossane	France	+	+	+	43	Sevillano	Spain	+	+	-
19	Kadesh	Israel	+	+	+	44	Sevillano2	Spain	+	+	-
20	Koroneiki	Greece	+	+	-	45	Sevillano3	Spain	+	+	+
21	Late Blanquette	Australia	+	+	-	46	Sevillano-lovisone	Unknown	+	+	+
22	Leccio	Italy	+	+	-	47	Souri	Palestine	+	+	-
23	Leccino	Italy	+	+	+	48	Tragolea	Greece	+	+	-
24	Lucca	Australia	+	+	-	49	Zitoum	Morocco	+	+	+

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OYLaV does not interfere negatively in oil yield and quality parameters

SHORT COMMUNICATION

Oil Viruses

European Journal of Lipid Science and Technology

www.ejlst.com

Impact of Olive Leaf Yellowing Associated Virus on Olive (*Olea europaea* L.) Oil

Anna Fontana, Amalia Piscopo, Alessandra De Bruno, Antonio Tiberini, Innocenzo Muzzalupo, and Giuliana Albanese*

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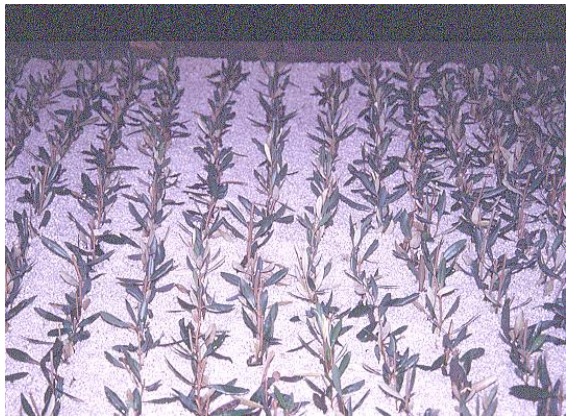
Fontana et al 2019

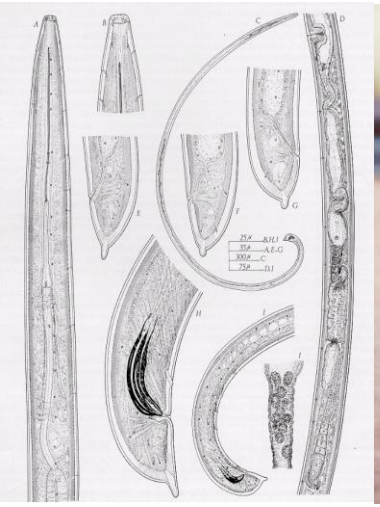
Spread of the viruses and virus-like diseases

- **Infected propagating material is the major means of virus dissemination**



Other possible means ??

- Seed transmission: OLV-1, CLRV
- Nematode vector: ArMV, SLRV
- Pollen: CLRV
- Aphid: CMV
- Psyllid, scale insects: OLYaV
- Leafhopper: Phytoplasmas
- Whiteflie: OEGV
- Without intervention of vector: OLV-1, TMV





Control

Virus Management – A Prevention Strategy

- The only control method is prevention
- Prevention is based on the use of VIRUS TESTED MATERIAL

➢ Plant disease-tested, certified propagative material

➢ Virus elimination

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European and Mediterranean Plant Protection Organization (EPPO)

European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes

PM 4/17 (2)

Schemes for the production of healthy plants for planting
Schémas pour la production de végétaux sains destines à la plantation

Pathogen-tested olive trees and rootstocks

Certification scheme for pathogen-tested trees and rootstocks of olive

Specific scope

This standard describes the production of certified pathogen-tested olive trees and rootstocks.

Specific approval and amendment

First approved in 1996-2009.
Revised in 2005-09.

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2006 OEPP/EPPO, Bulletin OEPP/EPPO Bulletin 36, 77-83

Viruses covered by the EPPO scheme

no.	Virus species	Genus
1	*Strawberry latent ring spot virus* (SLRSV)	Nepovirus
2	*Arabis mosaic virus* (ArMV)	Nepovirus
3	*Cherry leaf roll virus* (CLRV)	Nepovirus
4	*Cucumber mosaic virus* (CMV)	Cucumovirus
5	Olive leaf yellowing-associated virus (OLYaV)	Closterovirus

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Clean plant program: diagnosis of olive viruses

Symptomatology:

- Does not help much with diagnosis

Bioassays

- **Indexing:** not possible, no differential indicators
- **Mechanical transmission:** possible but unreliable

Laboratory methods

- **Serology:** ELISA
- **Molecular tools:** RT-PCR, dsRNA

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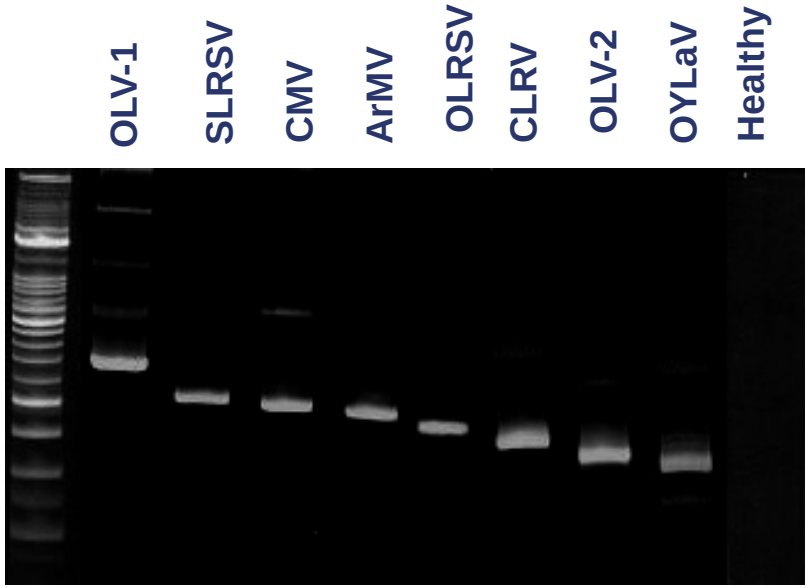
RT-PCR for the detection of 8 olive viruses



(Grieco *et al.*, 2000)

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Establishment of a Sensitive qPCR Methodology for Detection of the Olive-Infecting Viruses in Portuguese and Tunisian Orchards

Maria Doroteia Campos[1], Mohamed Salem Zellama[2], Carla Varanda[1], Patrick Materatski[1], Augusto Peixe[3], Maher Chaouachi[2] and Maria do Rosário Félix[3]*

[1] ICAAM – Instituto de Ciências Agrárias e Ambientais Mediterrânicas, Instituto de Investigação e Formação Avançada, Universidade de Évora, Évora, Portugal, [2] Laboratoire de Recherche "Bioressources: Biologie Intégrative & Valorisation," Institut Supérieur de Biotechnologie de Monastir, Université de Monastir, Monastir, Tunisia, [3] Departamento de Fitotecnia, ICAAM – Instituto de Ciências Agrárias e Ambientais Mediterrânicas, Escola de Ciências e Tecnologia, Universidade de Évora, Évora, Portugal

TABLE 2 | Primers used on qPCR assays.

Species	Accession ID	Primers (5′ → 3′)	AS (bp)	References
OMMV and/or TNV-D	AY616760	Fw: GTGTTCAGTCATATACATACC	247	Cardoso et al. (2004)
		Rv: GCCTATTGTGCTGTACCAC		
OLV-1	KF804054	Fw: GGGGTATGATGGTGCTATGG	162	This work
		Rv: ACTCCGCAATATCCGTTCTG		
OLYaV	AJ844555	Fw: GCTTATCTACTACGCCGATCTTGTC	71	This work
		Rv-AAGAGTGGATCCATCTAGATCGAAA		

Description of forward (Fw) and reverse (Rv) primers used for specific detection of Olive mild mosaic virus (OMMV) and/or Tobacco necrosis virus D (TNV-D), Olive latent virus 1 (OLV-1) and Olive leaf yellowing-associated virus (OLYaV), in the qPCR assays. AS, amplicon size.

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Campos et al. 2019

Objectives

1. Screen select olive tree populations for targeted viruses to compile a representative set of genome sequences (as complete as possible), and evaluate current published primers.

2. Incorporate new genetic data into a characterization of genetic variation across the targeted viruses to inform assay design.

3. Construct assays by targeting conserved domains and utilizing multiple primer sets as necessary to detect all known virus variants.

4. Empirically test and validate assays using positive controls.

5. Disseminate research progress and results.

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Objective 1: Screen olive tree populations for targeted viruses

- Obtain olive viruses and virus-like agents from infected plant material previously identified positive for:
  - Olea Europaea Geminivirus (OEGV)
  - Olive latent ring spot virus (OLRSV)
  - Olive latent virus 1 (OLV-1)
  - Olive latent virus 2 (OLV-2)
  - Olive latent virus 3 (OLV-3)
  - Olive leaf yellowing associated virus (OLYaV)
  - Olive mild mosaic virus (OMMV)
  - Tobacco necrosis virus D
- Surveys of USDA National Clonal Germplasm Repository (NCGR), local trees, and FPS olive trees have located trees infected with OEGV, OLV-3 and OLYaV.
- We will request positive control samples from colleagues in Europe for the remaining olive viruses.

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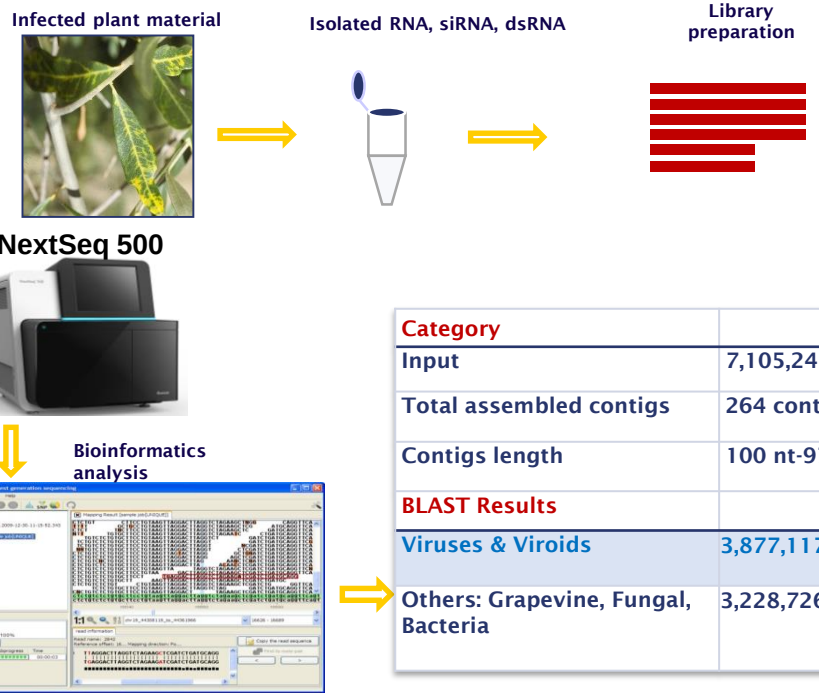
Objective 2: Incorporate new genetic data

High-throughput sequencing (HTS)

- Millions of short sequences (reads) generated for a given sample.
- Reads assembled into contiguous consensus sequences (contigs).
- Comprehensive picture of the entire microbial profile.
- Very sensitive: unknown viruses or very low titer asymptomatic species can be seen for the first time.



Category	
Input	7,105,243 Reads
Total assembled contigs	264 contigs
Contigs length	100 nt-9724 nt
BLAST Results	
Viruses & Viroids	3,877,117 Reads
Others: Grapevine, Fungal, Bacteria	3,228,726

- Contigs with a high affinity to plant viruses.

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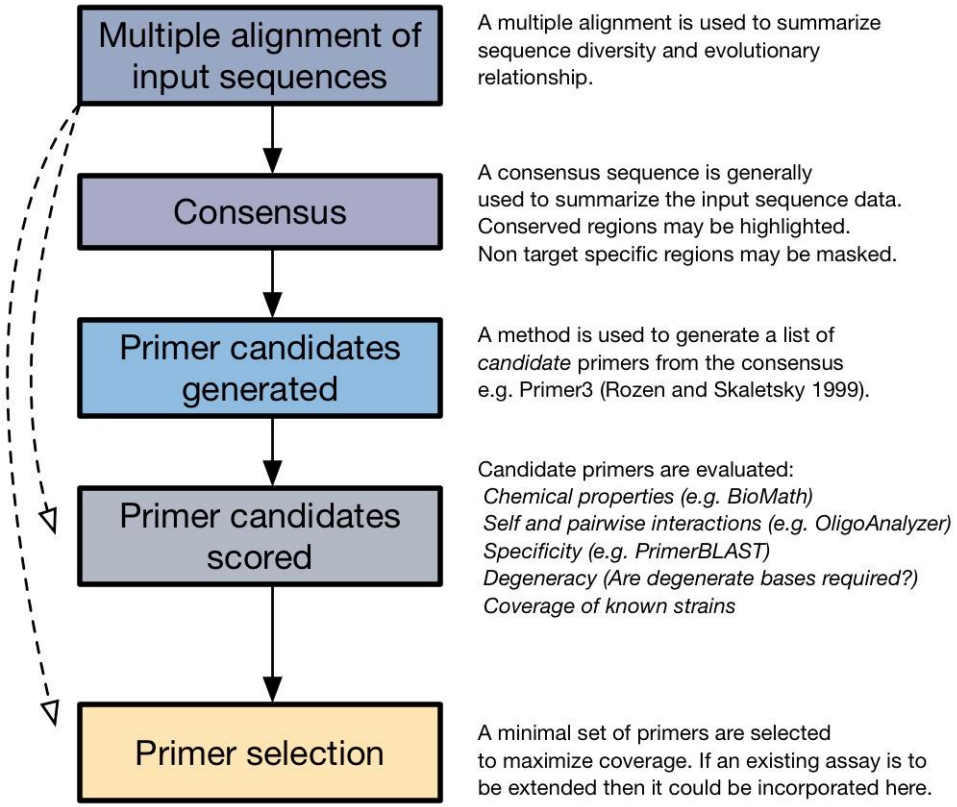
Objective 3: Construct improved assays

Design of novel qPCR assays

- Evaluate published assays
- Construct novel qPCR assays for:
  - OEGV
  - OLRSV
  - OLV-1
  - OLV-2
  - OLV-3
  - OLYaV
  - OMMV
  - TNV-D



Generic schematic for PCR detection assay design used by programs and web servers (e.g. Gadberry 2005; Duitama *et al.* 2009).

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Objective 3: Construct improved assays

- Sequence data available at GenBank or generated at FPS is aligned using a custom script developed in-house.
- Script identifies potential candidates for primers/probes.
- Candidate primers/probes are adjusted according to the parameter for TaqMan real-time PCR (MGB probes).
- Adjusted primers/probes are aligned to determine identity.

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Benefits of proposed project

- Development of robust, sensitive and reliable detection methods with a broad-range detection capacity is needed for large scale virus testing
- These assays will help clean stock programs and the olive industry with early virus detection at a lower cost
- Assays developed will be made available to CDFA and private commercial diagnostic labs
- Providing growers with virus tested olive materials will prevent virus introduction and spread

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USDA United States Department of Agriculture

United States Department of Agriculture
Animal and Plant Health Inspection Service
Plant Protection & Quarantine
4700 River Road
Riverdale, MD 20737

Controlled Import Permit to Import Restricted or Not Authorized Plant Material

Regulated by 7 CFR 319.6

- FPS has a new SOP using HTS and PCR
- HTS is required for release of material
- Reduces testing time frame to one year instead of two

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Timeline

Objectives	2021-2022
Collect virus sequences	X
Locate positive controls	X
Construct assays	X
Test assays	X
Disseminate results	X

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Budget for 2021-2022

BUDGET CATEGORY	Year 1
PERSONNEL: Salary and fringe benefits.	$46,260
TRAVEL	$2,000
MATERIALS & SUPPLIES	$15,000
TOTAL DIRECT COSTS	$63,260
Rate 10% (personnel only)	$4,626
TOTAL COSTS PER YEAR	**$67,886**

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Thank you, California Fruit Tree, Nut Tree, And Grapevine Improvement Advisory Board, for continued support of FPS